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Combinatorial targeting of NMDARs and 5-HT$_4$Rs exerts beneficial effects in a mouse model of Alzheimer's disease

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Abstract

Background Alzheimer's disease (AD) is the leading cause of dementia. There are limited approved medications that delay cognitive decline or lessen neuropsychiatric symptoms. Numerous clinical trials for AD using a single drug administration have failed to meet therapeutic endpoints, which is most likely due to the complexity of AD. A multimodal therapeutic intervention is more likely to improve symptoms by targeting multiple targets implicated in AD. Here, we investigated if targeting both N-Methyl-D-aspartic acid receptors (NMDARs) and serotonin type 4 receptors (5-HT$_4$R) may have beneficial effects in a mouse model of AD, as they have separately been shown to improve cognition and/or mood.

Methods Male and female control (Ctrl) or APP/PS1 mice were administered single, intermittent, or chronic administration of 1) saline; 2) (*R*,*S*)-ketamine, an NMDAR antagonist; 3) prucalopride, a 5-HT$_4$R agonist; or 4) (*R*,*S*)-ketamine + prucalopride to simultaneously target co-morbid neuropsychiatric and cognitive deficits. Behavioral assays were then administered to measure cognition, perseverative behavior, hyponeophagia, and/or sleep. Brains were processed for glial fibrillary acidic protein (GFAP) immunohistochemistry.

Results Single and chronic administration of (*R*,*S*)-ketamine + prucalopride administration improved cognitive decline by increasing memory retrieval in a contextual fear conditioning (CFC) paradigm in APP/PS1 mice. Drug efficacy was less effective in females than in males and was age dependent. Hippocampal GFAP immunoreactivity was decreased by chronic (*R*,*S*)-ketamine + prucalopride treatment in females.

Conclusions Our results indicate that combined administration of (*R*,*S*)-ketamine + prucalopride is a novel multimodal therapeutic strategy to treat cognitive decline in AD. Future work will further characterize these interactions with the goal of clinical development.

Keywords Neurodegeneration, Adjunctive treatment, Ketamine, Prucalopride, Neuroinflammation

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Background

While Alzheimer's disease (AD), a neurodegenerative disorder leading to cognitive decline, was discovered 110 years ago, there are only a handful of approved drugs for symptomatic treatment [1–3]. As of April 2025, the National Institute on Aging (NIA) supports 495 active clinical trials; however, many clinical trials of single drug administration have failed to meet endpoints [4]. These data suggest that the complex pathophysiology of AD may necessitate combinatorial treatments rather than a single drug therapy.

Neuropsychiatric symptoms (NPS), such as depression and anxiety, have become a focus of numerous studies as early biomarkers and treatment targets for AD [5–8]. However, there are currently only 3 clinical trials targeting NPS in AD [4]. One of the biggest breakthroughs in the study of depression has been the discovery of (*R*,*S*)-ketamine as a rapid-acting antidepressant [9, 10]. (*R*,*S*)-ketamine is an *N*-Methyl-D-aspartic acid receptor (NMDAR) antagonist initially developed as an anesthetic, has antidepressant effects as rapidly as 2 h after administration, and exerts efficacy for up to 6 weeks after a single administration [9–12]. Interestingly, memantine, the most prescribed drug for AD, is also a non-competitive NMDAR antagonist [13]. While memantine has fewer side-effects than (*R*,*S*)-ketamine, it is only modestly effective in improving memory loss in AD [14]. Researchers have discussed using (*R*,*S*)-ketamine as a potential treatment for AD, but to date there is no registered clinical trial administering (*R*,*S*)-ketamine to AD subjects [15]. Additionally, within the depression field, concerns of non-specific adverse side effects (e.g., cardiovascular, neurological, or cognitive side effects) and abuse potential may have previously limited therapeutic utility [16–18].

In addition to NMDARs, serotonin type 4 receptors (5-HT_4Rs) have become of interest as therapeutic agents for AD. 5-HT_4R activation stimulates α-cleavage of the amyloid precursor protein (APP), leading to the release of soluble and neurotrophic sAPPα fragments [19, 20]. In the brain, activation of 5-HT_4Rs increases neuronal firing, increases neurogenesis, and can protect against as well as treat depression and anxiety [21–26]. Numerous studies have also shown that 5-HT_4R agonists are nootropic due to their ability to enhance learning and memory in rodent models [27–41]. Prucalopride, also known as Motegrity®, has recently gained some attention, as it is the only currently available Food and Drug Administration (FDA)-approved 5-HT_4R agonist. Although prucalopride is indicated for the treatment of chronic idiopathic constipation (CIC), several studies show that it may exert beneficial effects in multiple rodent models of AD, such as enhancing the excitability of hippocampal CA1 neurons [42], attenuating aggregation of tau protein [43], and enhancing sAPPα production [44]. However, there is a gap in knowledge in determining if combined drug administration targeting NMDARs and 5-HT_4Rs could have therapeutic effects on both mood and cognition in AD.

Here, we tested the behavioral and astrocytic effects of (*R*,*S*)-ketamine and prucalopride in a mouse model of AD. Mice were administered at varying doses either: 1) saline; 2) (*R*,*S*)-ketamine; 3) prucalopride; or 4) (*R*,*S*)-ketamine + prucalopride to simultaneously target co-morbid neuropsychiatric and cognitive deficits in Control (Ctrl) or APP/PS1 mice. Assays were then administered to measure cognition, perseverative behavior, behavioral despair, hyponeophagia, and/or sleep. Single or chronic combined (*R*,*S*)-ketamine + prucalopride administration improved memory retrieval in APP/PS1 mice. Chronic (*R*,*S*)-ketamine + prucalopride administration restored hippocampal GFAP expression in female APP/PS1 mice comparable to normal Ctrl expression. Our results indicate that combined administration of (*R*,*S*)-ketamine + prucalopride could be a novel multimodal therapeutic strategy to enhance cognitive impairment and reduce astrogliosis in AD.

Methods

For a full description of Methods and Materials, please refer to the Supplemental Methods in Supplement 1 and Table S01.

Mice

The APP/PS1 (AD) mice were maintained on a 129S6/SvEv background as previously described [5, 45–47]. Female and male mice were used in all experiments. Mice were aged to either 2, 6, 6–9, or 12 months of age for specific experiments as described below. Mice were group housed (4–5 per cage) in a 12-h light/dark cycle (lights on at 0600 h) colony room maintained at 22 ± 2°C. Mice had ad libitum access to food and water. All experiments were approved by the Institutional Animal Care and Use Committees (IACUCs) at Columbia University Irving Medical Center (CUIMC) (CUIMC IACUC Protocol #AC-AABO9556) and at the Research Foundation of Mental Hygiene, Inc. (RFMH) at the New York State Psychiatric Institute (NYSPI) (NYSPI IACUC Protocol #1620).

Drugs

Single injection

Saline (Sal) (0.9% NaCl), (*R*,*S*)-ketamine (K) (Ketaset, Zoetis, Parsippany-Troy Hills, NJ), prucalopride (P) (SML1371, Sigma-Aldrich, St. Louis, MO), or combined (*R*,*S*)-ketamine + prucalopride (K + P) was administered once prior to sleep experiments. All drugs were prepared

in physiological saline and administered intraperitoneally (i.p.) in volumes of 0.1 cc per 10 grams (g) body weight [23, 48].

Chronic injections

Drugs were prepared as previously described [24]. Sal and P (1.5 or 3 mg/kilogram (kg)) were administered daily. K (10 or 30 mg/kg) was administered once or twice per week. For combined K + P, K was administered once per week, consistent with previous studies [49, 50], and P was administered daily. K was administered, at most, twice per week to avoid inducing schizophrenia-like behaviors in mice [51]. To control for administration stress, mice were administered Sal on days when K was not administered.

Statistical analysis

Data were analyzed using Prism 10.4.0 (Graphpad Software, La Jolla, CA). Alpha was set to 0.05 for all analyses. The effect of Genotype or Drug was analyzed using either a one- or two-way analysis of variance (ANOVA), using repeated measures where appropriate. Post-hoc Dunnett, Sidak, or Tukey tests were used where appropriate. All statistical tests and *p* values are listed in Table S02.

Results

A single dose of combined (*R,S*)-ketamine + prucalopride improves memory retrieval in male APP/PS1 mice

We first sought to determine if combined K + P would alter sleep behavior in 6- to 9-month-old Ctrl and APP/PS1 male mice (Fig. 1A). Following drug administration, mice were placed in Piezo sleep boxes for 5 days. Average activity was measured across 24 h throughout the light (0–12 zeitgeiber (ZT)) and dark periods (12–23 ZT). As expected, mice exhibited increased activity during the night. However, activity was decreased significantly in the combined K + P-administered APP/PS1 mice at 12–14 ZT in comparison to Sal-administered APP/PS1 mice (Fig. 1B-C). Nonetheless, overall sleep and amplitude, measures of sleep and wake differences, were not significantly impacted by drug administration (Fig. 1D-E). During the light phase, K (10 mg/kg) and K + P (10 + 3 mg/kg) reduced sleep in APP/PS1 mice when compared with Ctrl mice (Fig. 1F). Additionally, in APP/PS1 mice, K (10 mg/kg) reduced sleep in comparison to APP/PS1 Sal-administered mice (Fig. 1F). There was no effect of Drug or Genotype during the dark phase (Fig. 1G). These data suggest that combined K + P does not significantly alter sleep/wake behaviors.

Mice were next tested in a battery of behavioral paradigms to measure hyponeophagia, perseverative behavior, and memory. Mice were not re-administered i.p. drug injections prior to these behavioral assays. In the novelty suppressed feeding (NSF) paradigm, behavior was comparable across all groups in the novel arena (Fig. 1H-L). However, in the home cage, K (30 mg/kg) increased latency to feed in APP/PS1 mice when compared with saline (Fig. S1A-S1C). In the marble burying (MB) task, APP/PS1 Sal-administered mice buried significantly less marbles than Ctrl Sal-administered mice, but there was no effect of drug administration (Fig. 1M). Lastly, mice were administered a 3-shock CFC paradigm to assay learning and memory. During training, P (3 mg/kg) increased freezing in APP/PS1 mice when compared to Sal-administered APP/PS1 mice (Fig. S1D-S1F). During memory retrieval, in Ctrl mice, K (30 mg/kg) increased overall freezing in comparison to Sal; however, average freezing was comparable across all groups of Ctrl mice (Fig. 1N, P). Notably, K (30 mg/kg) and K + P (10 + 3 mg/kg) increased freezing behavior, a proxy for memory retrieval, in APP/PS1 mice (Fig. 1O-P). These data suggest that K and combined K + P does not impact NPS but could be effective for improving cognition in AD. Notably, the dose of K that was necessary to induce a behavioral effect was significantly higher when administered alone (30 mg/kg) in comparison to when administered in combination with P (10 mg/kg). Overall, this finding suggests that combinatorial K + P may allow for decreased dosing of K.

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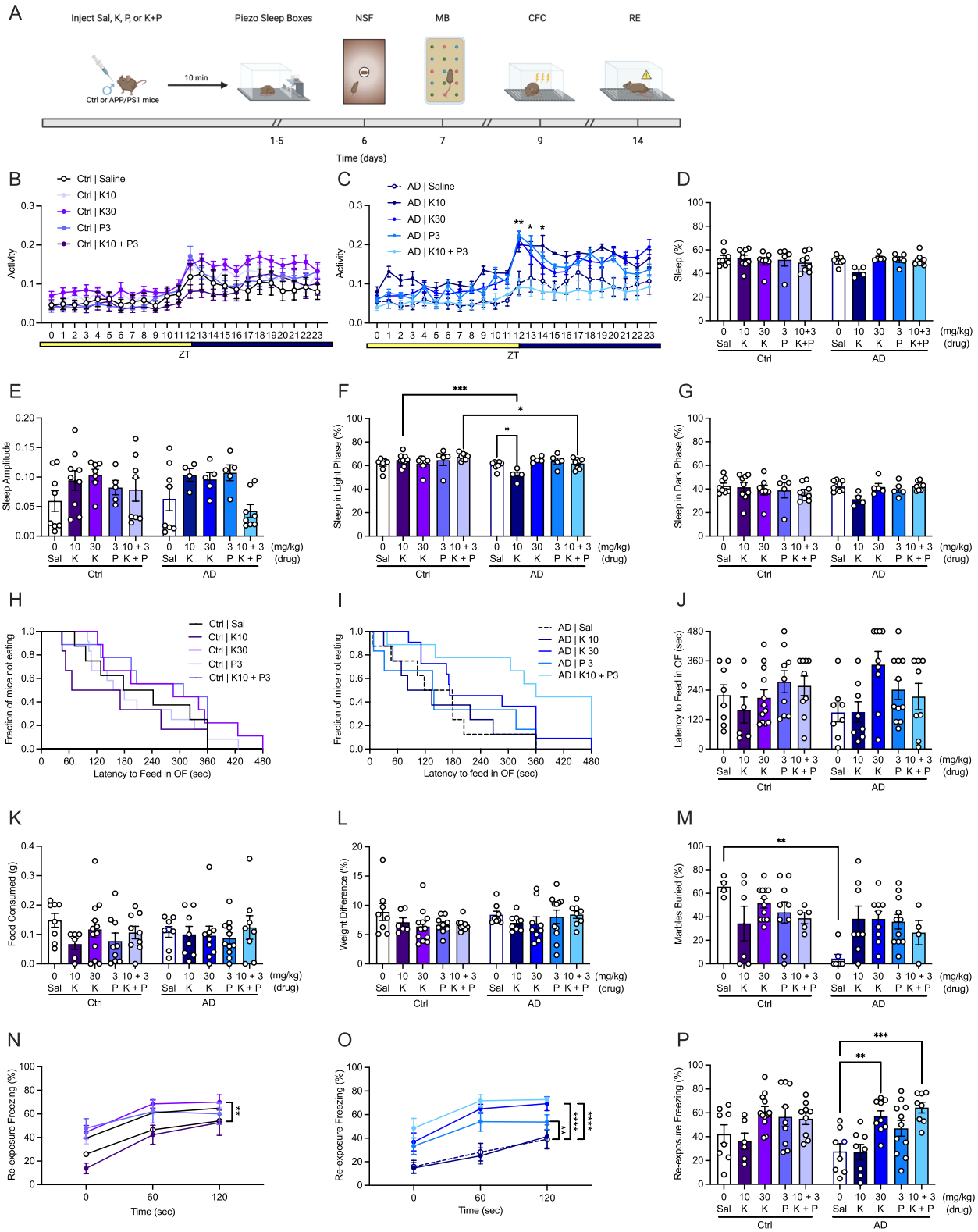
Fig. 1 A single administration of combined (*R,S*)-ketamine + prucalopride improves memory retrieval in male APP/PS1 mice. **A** Experimental design. **B-C** Mice generally exhibited increased activity at night. In APP/PS1 mice, at ZT 12–14, K + P (10 + 3 mg/kg)-administered mice exhibited reduced activity in relation to other experimental groups. **D-E** However, overall sleep and sleep amplitude were comparable across all groups. **F** During the light phase, K (10 mg/kg)-administered APP/PS1 mice unexpectedly exhibited less overall sleep compared to saline-administered APP/PS1 and K (10 mg/kg)-administered Ctrl mice. K + P-administered APP/PS1 mice also exhibited increased sleep relative to their respective Ctrl group. **G** There was no effect of Drug or Genotype in the dark phase. **H–L** Behavior in the NSF assay was similar in all groups. **M** Sal-administered APP/PS1 mice buried significantly less marbles in relation to Sal-administered Ctrl mice. **N-P** K (30 mg/kg) and combined K + P (10 + 3 mg/kg)-administered APP/PS1 mice exhibited significantly increased freezing in comparison to Sal-administered APP/PS1 mice. ($n = 4$–12 male mice per group). Error bars represent ± SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. Sal, saline; K, (*R,S*)-ketamine; P, prucalopride; NSF, novelty suppressed feeding; MB, marble burying; CFC, contextual fear conditioning; Ctrl, control; AD, Alzheimer's disease; ZT, zeitgeiber; mg, milligram; kg, kilogram; OF, open field; sec, seconds; g, grams



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A single dose of combined (*R,S*)-ketamine + prucalopride does not alter behavior in female APP/PS1 mice

Next, we sought to test the efficacy of combined K + P in Ctrl and APP/PS1 female mice. Female mice were administered a single dose of Sal, K, P, or K + P prior to sleep and behavioral testing (Fig. 2A). Across all days, average activity and overall sleep was comparable between genotypes and drug groups (Fig. 2B-D). In Ctrl mice, K (10 mg/kg) and P (1.5 mg/kg) significantly reduced sleep amplitude relative to Sal-administered mice (Fig. 2E). However, there was no effect of Drug or Genotype in sleep during the light or dark phase (Fig. 2F-G). Next, mice were administered a series of behavioral assays. Mice were not given additional i.p. drug injections prior to these behavioral analyses. In the NSF, a Mantel-Cox test indicated that there was a trending effect of Drug on latency to feed in Ctrl, but not APP/PS1 mice (Fig. 2H-I). However, mean latency to feed, food consumed and change in weight was similar across all groups (Fig. 2J-L). During the home cage portion of the task, there was a significant effect of Drug in Ctrl, but not APP/PS1 mice (Fig. S2A-S2B). However, when analyzed with a 2-way ANOVA, there was no significant effect of Genotype or Drug on latency to feed in the home cage (Fig. S2C). In the MB and CFC assays, behavior was comparable across all groups (Fig. 2M-P, S2D-S2F). Overall, these data suggest that combined K + P does not significantly impact behavior in a female APP/PS1 mice.

Chronic combined (*R,S*)-ketamine + prucalopride administration increases memory retrieval in 2-month-old male APP/PS1 mice

Next, we aimed to determine whether chronic administration of K + P could enhance cognitive function or reduce NPS in APP/PS1 mice. As AD is a chronic neurodegenerative disorder, it is likely that repeated pharmacological treatment is a more viable treatment option. Additionally, early treatment of AD could significantly improve quality of life and life expectancy for AD patients, and preventing early cognitive decline could be important for reducing symptoms in later stages of the illness [52–54]. Therefore, we aimed to test whether chronic administration of combined K + P improved memory or altered additional behaviors in mice at 2 months of age.

Starting at 2 months of age, Ctrl and APP/PS1 mice were given chronic i.p. injections of saline, K, P, or K + P at varying doses and frequencies for 2 weeks prior to 3-shock CFC (Fig. 3A). Mice were then tested in CFC, forced swim test (FST), and MB assays. During drug administration, change in body weight was comparable across all groups, indicating that chronic drug administration does not exert nonspecific effects on body weight (Fig. 3B). While there was a significant effect of Drug during minutes 4 and 5 of CFC training in APP/PS1 mice (Fig. S3A-S3B), there was no significant effect of Drug or Genotype on average freezing during (Fig. 3C). However, during CFC re-exposure, in APP/PS1 mice, P (3 mg/kg, 7X) and K + P (10 mg/kg, 1X + 3 mg/kg, 7X) increased average freezing when compared with Sal (Fig. 3D, S3C-S3D). On FST day 1, K (10 mg/kg, 2X) reduced average immobility time in APP/PS1 mice when compared with the respective Ctrl mice (Fig. 3E, S3E-S3F), but there was no significant effect of Drug or Genotype on FST day 2 (Fig. 3F, S3G-S3H). In the MB assay, K (10 mg/kg, 2X), K (30 mg/kg, 2X), P (3 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) reduced marble burying in APP/PS1 mice when compared to respective Ctrl mice (Fig. 3G). In contrast, K (30 mg/kg, 1X) increased marble burying in APP/PS1 mice when compared to the respective Ctrl group. Overall, these results suggest that chronic K + P enhances memory retrieval in male APP/PS1 mice.

Subsequently, we repeated the same experiment in female APP/PS1 mice at 2 months of age (Fig. S4A). Changes in body weight were comparable across all groups, indicating that chronic drug administration does not significantly alter body weight in female mice (Fig. S4B). During CFC training, average freezing was significantly reduced in APP/PS1 mice administered Sal, K (10 mg/kg, 2X), P (1.5 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) when compared to respective Ctrl mice (Fig. S4C). However, during CFC re-exposure, there was no significant effect of Genotype or Drug on average freezing (Fig. S4D). On FST day 1, K (10 mg/kg, 1X), but no other drug groups, significantly reduced average immobility in APP/PS1 mice when compared to Sal APP/

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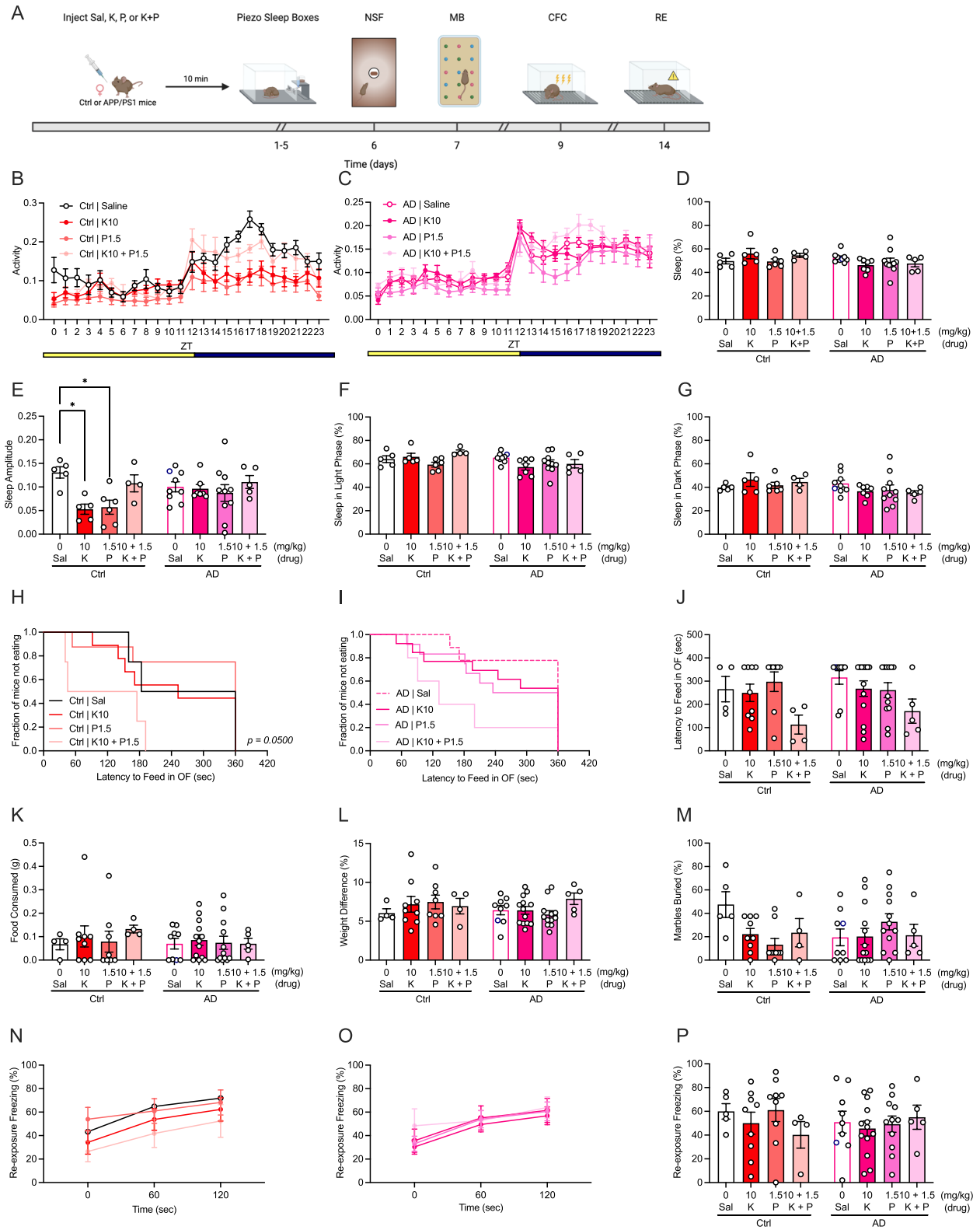
Fig. 2 Single administration of (*R,S*)-ketamine + prucalopride does not significantly impact behavior in female APP/PS1 mice. **A** Behavioral paradigm. **B-D** Activity and overall sleep were not significantly impacted by drug treatment in Ctrl and APP/PS1 mice. **E** K (10 mg/kg) and P (1.5 mg/kg), but not K + P administration, reduced sleep amplitude in Ctrl mice relative to Sal. **F-G** However, sleep in the light and dark phase were not altered across drug and genotype groups. **H-I** There was a trending ($p = 0.0500$), but not significant, effect of Drug on latency to feed in the novel arena in Ctrl, but not APP/PS1 mice during the NSF. All other behaviors in the (**J-L**) NSF, (**M**) MB, and (**N-P**) CFC assays were comparable across all groups. ($n = 4$–13 female mice per group). Error bars represent ± SEM. * $p < 0.05$. Sal, saline; K, (*R,S*)-ketamine; P, prucalopride; Ctrl, control; AD, Alzheimer's disease; NSF, novelty suppressed feeding; MB, marble burying; CFC, contextual fear conditioning; ZT, zeitgeiber; mg, milligram; kg, kilogram; OF, open field; sec, seconds; g, grams



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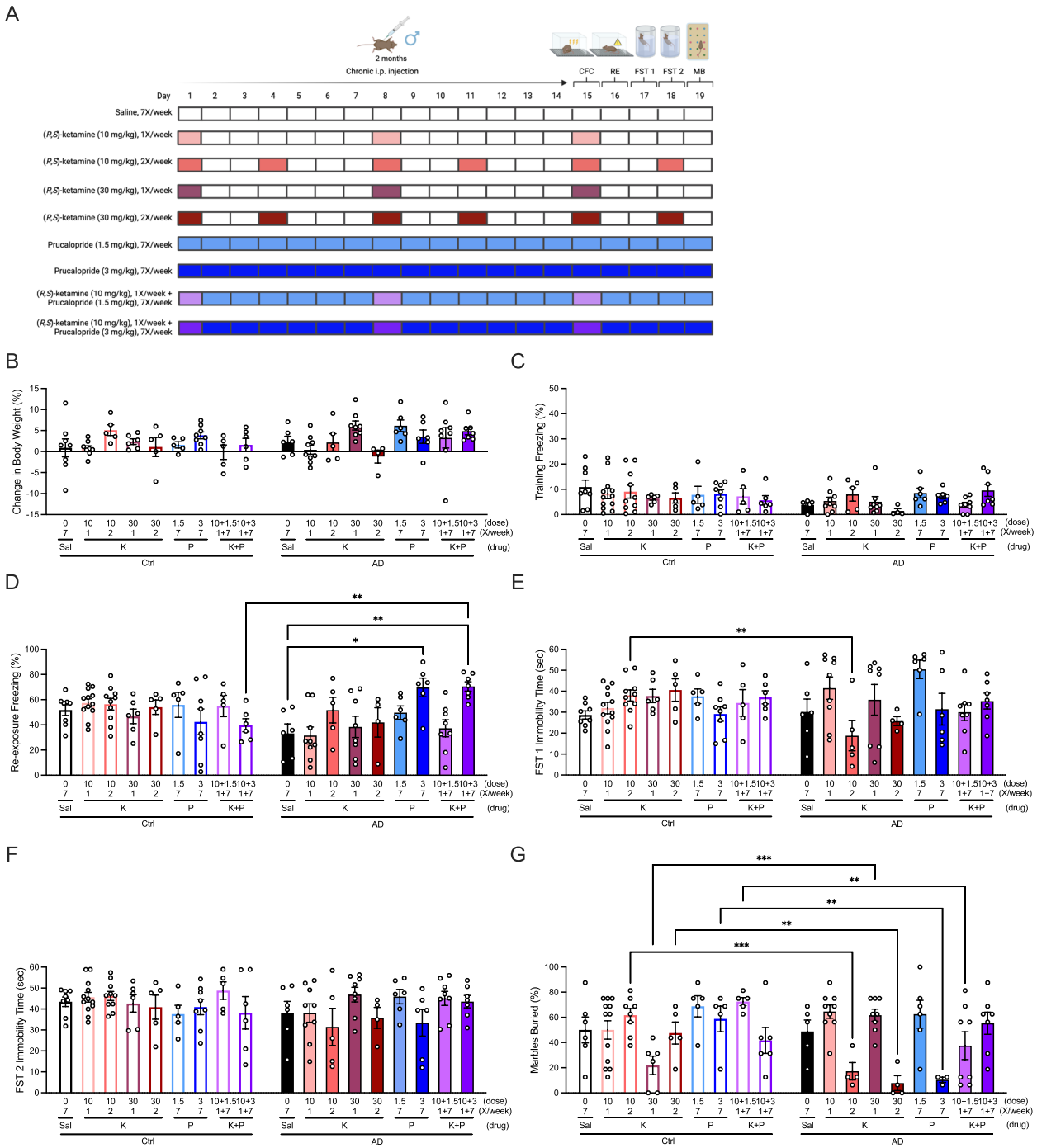

Fig. 3 Chronic, combined (*R,S*)-ketamine + prucalopride enhances memory retrieval in 2-month-old male APP/PS1 mice. **A** Behavioral paradigm. **B** Chronic drug administration did not significantly affect body weight**. C** Freezing during CFC training was comparable across all groups. **D** However, during CFC re-exposure, combined K + P (10 mg/kg, 1X + 3 mg/kg, 7X) significantly increased freezing in APP/PS1 mice in relation to K + P (10 mg/kg, 1X + 3 mg/kg, 7X)-administered Ctrl and Sal-administered APP/PS1 mice. In addition, P (3 mg/kg, 7X) also significantly increased freezing relative to Sal-administered APP/PS1 mice. **E** K (10 mg/kg, 2X) reduced immobility time in APP/PS1, but not Ctrl mice during FST day 1. **F** Behavior during FST day 2 was not significantly impacted by Drug treatment. **G** K (10 mg/kg, 2X), K (30 mg/kg, 1X), K (30 mg/kg, 2X), P (3 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) significantly reduced marbles buried in APP/PS1 mice relative to Ctrl mice. ($n = 5$–13 2-month-old male mice per group). Error bars represent ± SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. i.p., intraperitoneal; CFC, contextual fear conditioning; RE, re-exposure; FST, forced swim test; MB, marble burying; Sal, saline; K, (*R,S*)-ketamine; K + P, (*R,S*)-ketamine + prucalopride; X, times; Ctrl, control; AD, Alzheimer's disease; sec, seconds

PS1 mice (Fig. S4E). In the FST day 2 and MB assays, behavior was comparable across all groups (Fig. S4F-S4G). These findings suggest that while K + P is effective in enhancing memory retrieval in male 2-month-old APP/PS1 mice, it does not significantly alter behavior in 2-month-old APP/PS1 female mice. More dosing combinations or increased length of administration may be necessary for female APP/PS1 mice.

Chronic combined (*R,S*)-ketamine + prucalopride administration improves memory retrieval in 6-month-old male, but not female APP/PS1 mice

We then aimed to determine whether chronic administration of combined K + P was sufficient to enhance cognitive function or reduce NPS in older APP/PS1 mice. We administered the same chronic drug administration and behavioral testing paradigm as detailed in Fig. 3, with the exception that 6-month-old male Ctrl and APP/PS1 mice were used for testing (Fig. 4A).

Changes in body weight were not significantly impacted by chronic drug administration in 6-month-old male Ctrl and APP/PS1 mice (Fig. 4B). During CFC training, K (30 mg/kg, 2X) increased freezing in Ctrl mice when compared with Sal-administered Ctrl mice and K (30 mg/kg, 2X)-administered APP/PS1 mice (Fig. 4C, S5A-S5B). K + P (10 mg/kg, 1X + 3 mg/kg, 7X) also increased freezing in APP/PS1 mice in comparison to Ctrl mice. During CFC re-exposure, Sal-administered APP/PS1 mice displayed significantly reduced freezing when compared to Sal-administered Ctrl mice, indicating a deficit in memory retrieval as we have previously published (Fig. 4D, S5C-S5D) [5, 46, 47]. This deficit was rescued by chronic treatment with K + P (10 mg/kg, 1X + 3 mg/kg, 7X). Additionally, K (30 mg/kg, 2X) and P (3 mg/kg, 7X) reduced freezing in APP/PS1, but not Ctrl mice. During FST day 1, there was a significant effect of Genotype on average immobility time (Fig. 4E). While Sal-administered APP/PS1 mice had reduced immobility time in comparison to Sal-administered Ctrl mice, K (10 mg/kg, 2X) and K + P (10 mg/kg, 1X + 3 mg/kg, 7X) increased immobility in APP/PS1 mice relative to Ctrl groups (Fig. 4E, Fig. S5E-S5F). On FST day 2, in Ctrl mice, K (10 mg/kg, 2X), P (1.5 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) reduced immobility time when compared with Sal (Fig. 4F, Fig. S5G-S5H). K (10 mg/kg, 2X) and K (30 mg/kg, 1X) increased immobility in APP/PS1 mice when compared with Ctrl mice, while K + P (10 mg/kg, 1X + 3 mg/kg, 7X) decreased immobility in APP/PS1 but not Ctrl mice (Fig. 4F). In the MB assay, K + P (10 mg/kg, 1X + 3 mg/kg, 7X) reduced marbles buried in APP/PS1 mice when compared to the respective Ctrl mice (Fig. 4G). Overall, these results suggest that combined K + P improves memory retrieval in 6-month-old male APP/PS1 mice.

We then tested whether combined K + P was efficacious in 6-month-old APP/PS1 female mice by administering the same drug administration and behavioral testing schedule used in male mice (Fig. S6A). K (30 mg/kg, 1X) reduced weight in APP/PS1 mice when compared to Ctrl mice (Fig. S6B). During CFC training, freezing was comparable across all groups (Fig. S6C). During CFC re-exposure, Sal, K (30 mg/kg, 1X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) reduced freezing in APP/PS1 mice relative to Ctrl groups (Fig. S6D). During FST day 1, P (1.5 mg/kg, 7X) increased immobility in APP/PS1 when compared to the respective Ctrl mice (Fig. S6E). On FST day 2, in Ctrl mice, K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) significantly reduced immobility when compared to saline-administered Ctrl mice. Sal-administered APP/PS1 mice had reduced immobility when compared to Sal-administered Ctrl mice; conversely, K (10 mg/kg, 2X), K (30 mg/kg, 2X), P (1.5 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) significantly altered immobility in APP/PS1 mice in comparison to Ctrl mice (Fig. S6F). There was no significant effect of Drug or Genotype during the MB assay (Fig. S6G). Altogether, these findings suggest that while combined K + P improves memory retrieval in 6-month-old APP/PS1 male mice, it does not significantly rescue behavior in 6-month-old APP/PS1 female mice.

Chronic, combined (*R,S*)-ketamine + prucalopride administration alters GFAP expression in 6-month-old female, but not male APP/PS1 mice

To determine a potential mechanism by which combined K + P may exert its memory enhancing effects, we then used immunohistochemical techniques to measure glial acidic fibrillary protein (GFAP) expression in the hippocampus (HPC). GFAP is an astrocytic cytoskeletal protein that has been proposed as a pathological marker for the diagnosis and progression of AD [55–60]. In particular, GFAP is an indicator of activated astrocytes, which may be stimulated in the areas surrounding Aβ plaques or by the accumulation of tau protein [61, 62]. Additionally, GFAP may be activated in areas of high neuroinflammation [63].

Mice from cohorts in Fig. 4 and Fig. S6 were euthanized and brains were collected following the MB assay. Brain sections from mice administered Sal, K, P, or K + P at the effective doses were used to quantify GFAP in the HPC, dentate gyrus granule cell layer (DG-sg), dentate gyrus molecular layer (DG-mo), field CA1 stratum radiatum (CA1), and stratum lacunosum-moleculare (SLM) (Fig. 5A-B). In the HPC, P-administered Ctrl mice exhibited higher GFAP expression in comparison

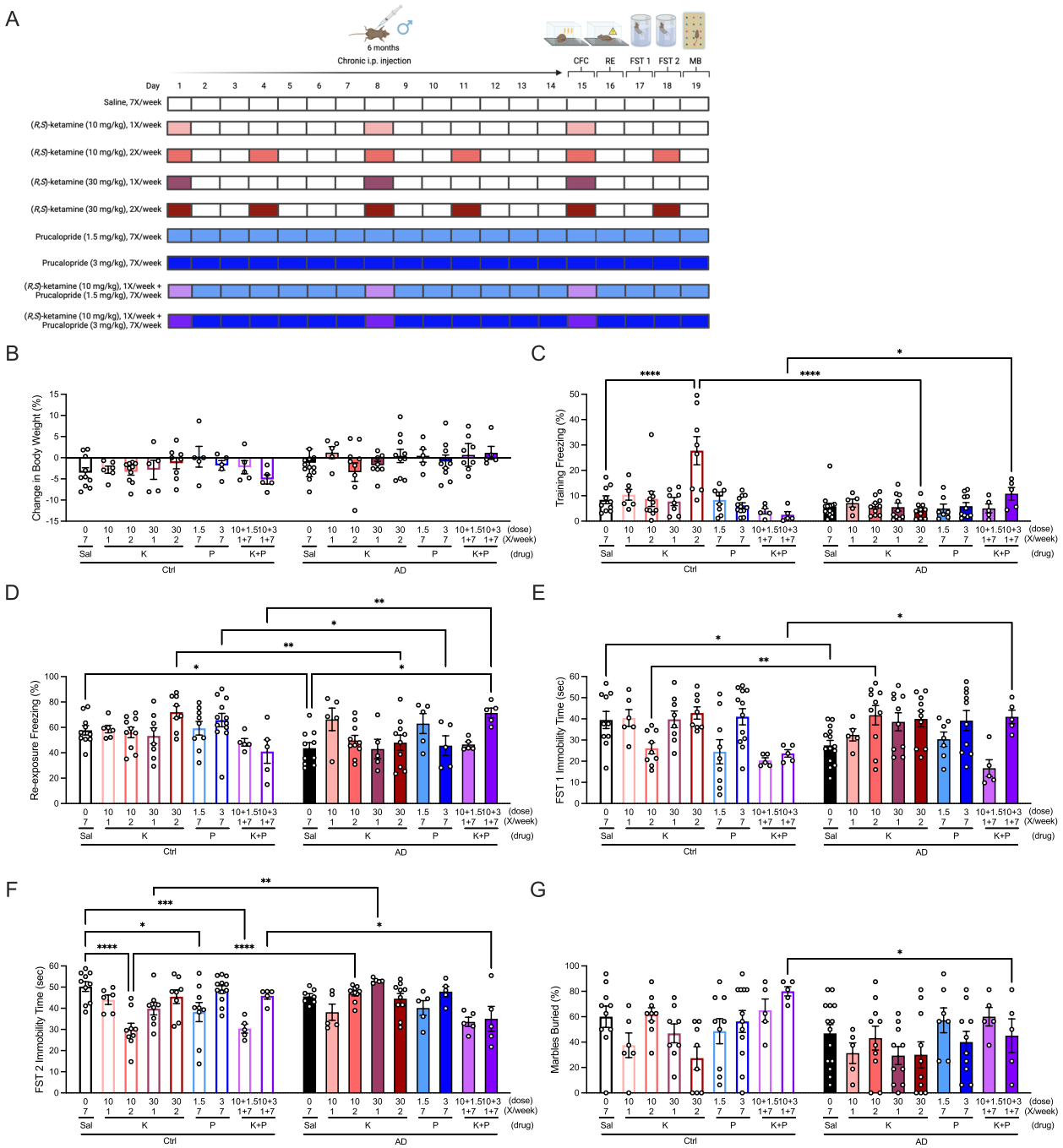

Fig. 4 Chronic, combined (*R,S*)-ketamine + prucalopride increases memory retrieval in 6-month-old male APP/PS1 mice. **A** Behavioral paradigm. **B** Body weight was comparable across all groups. **C** In Ctrl mice, K (30 mg/kg, 2X) increased freezing relative to saline-administered Ctrl and K (30 mg/kg, 2X)-administered APP/PS1 mice. K + P (10 mg/kg, 1X + 3 mg/kg, 7X) enhanced freezing in APP/PS1 mice compared to its respective Ctrl group. **D** Sal-administered APP/PS1 male mice exhibited reduced freezing relative to saline-administered Ctrl mice. This deficit in freezing was rescued by administration of K + P (10 mg/kg, 1X + 3 mg/kg, 7X). K (30 mg/kg, 2X), P (3 mg/kg, 7X), and K + P (10 mg/kg, 1X + 3 mg/kg, 7X) increased freezing in APP/PS1 mice relative to respective Ctrl groups. **E** On FST day 1, Sal-administered APP/PS1 mice had less immobility than Sal-administered Ctrl mice. K (10 mg/kg, 2X) and K + P (10 mg/kg, 1X + 3 mg/kg, 7X) increased immobility in APP/PS1 mice in comparison to respective Ctrl groups. **F** On FST day 2, in Ctrl mice, K (10 mg/kg, 2X), P (1.5 mg/kg, 7X), and K + P (10 mg/kg, 1X + 1.5 mg/kg, 7X) reduced immobility time compared to saline. K (10 mg/kg, 2X) and K (30 mg/kg, 1X) increased immobility in APP/PS1 mice compared to respective Ctrl groups, while K + P (10 mg/kg, 1X + 3 mg/kg, 7X) reduced immobility time in APP/PS1 mice compared to K + P-administered Ctrl mice. **G** K + P (10 mg/kg, 1X + 3 mg/kg, 7X) reduced marble burying in APP/PS1 mice relative to K + P-administered Ctrl mice. ($n = 5$–11 6-month-old male mice per group). Error bars represent ± SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. i.p., intraperitoneal; CFC, contextual fear conditioning; RE, re-exposure, FST, forced swim test; MB, marble burying; X, times; Sal, saline; K, (*R,S*)-ketamine; P, prucalopride; K + P, (*R,S*)-ketamine + prucalopride; AD, Alzheimer's disease; sec, seconds

to saline-administered Ctrl and P-administered APP/PS1 mice (Fig. 5C). In APP/PS1 male mice, K increased GFAP expression relative to Sal (Fig. 5C). In the HPC of female mice, Sal-administered APP/PS1 mice had significantly higher levels of GFAP in comparison to Sal-administered Ctrl mice. This increase was rescued by chronic K + P, but not K or P alone, in APP/PS1 mice, comparable to levels observed in Ctrl mice (Fig. 5D). P-administered APP/PS1 mice had higher GFAP levels relative to P-administered Ctrl mice (Fig. 5D). In the DG-sg, P-administered Ctrl mice had significantly increased GFAP expression in comparison to Sal-administered Ctrl and P-administered APP/PS1 mice (Fig. 5E). In the DG-sg of Ctrl female mice, there was an overall effect of Drug but not Genotype (Fig. 5F). In the DG-mo of male mice, K significantly increased GFAP in APP/PS1 mice in comparison to Sal-administered APP/PS1 mice and K-administered Ctrl mice (Fig. 5G). In the DG-mo of female mice, Sal-administered APP/PS1 mice exhibited increased GFAP in comparison to Ctrl mice, and this increase was rescued by K + P, but not K or P alone (Fig. 5H). Additionally, P administration increased GFAP in APP/PS1 mice in comparison to its respective Ctrl group (Fig. 5H). In the SLM of Ctrl male mice, P significantly enhanced GFAP expression relative to Sal- and P-administered APP/PS1 mice (Fig. 5I). In the SLM of female mice, there was an overall effect of Drug but not of Genotype (Fig. 5J). In CA1 of male Ctrl mice, P significantly increased GFAP relative to Sal-administered Ctrl mice and P-administered APP/PS1 mice, while K increased GFAP relative to Sal-administered APP/PS1 mice (Fig. 5K). Finally, in CA1 of female mice, K-administered APP/PS1 mice exhibited increased GFAP relative to Sal-administered APP/PS1 mice and K-administered Ctrl mice (Fig. 5L). Together, these findings suggest that 1) female APP/PS1 mice have significantly more hippocampal GFAP expression than female Ctrl mice; 2) K + P effectively rescues this increased hippocampal GFAP expression in female APP/PS1 mice; and 3) these effects in female HPC are likely driven specifically by the DG-mo region.

Chronic, combined (*R,S*)-ketamine + prucalopride administration does not significantly alter behavior in aged mice

Finally, we sought to test whether combined K + P could be effective in enhancing memory retrieval or reducing NPS in aged 12-month-old mice. Ctrl mice at 12 months of age were administered chronic Sal, K (10 mg/kg, 2X), P (1.5 mg/kg, 7X), or K + P (10 mg/kg, 2X + 1.5 mg/kg, 7X or 10 mg/kg, 2X + 3 mg/kg, 7X) for 2 weeks prior to CFC training and behavioral testing (Fig. S7A). During CFC training and re-exposure, freezing levels were comparable across all drug groups (Fig. S7B-S7E). On FST day 1, there was no significant impact of drug administration on immobility levels (Fig. S7F-S7G). On FST day 2, there was a significant effect of Drug on overall immobility, but average immobility levels were comparable between groups (Fig. S7H-S7I). Behavior during the MB assay as well as changes in body weight were not significantly altered by drug administration (Fig. S7J-S7L). Together, these results suggest that chronic drug administration does not significantly affect behavior in 12-month-old mice. Overall, our findings indicate that K + P drug administration is most effective in enhancing memory retrieval when administered earlier than 12 months of age.

Discussion

In this study, we characterized the effects of acute and chronic administration of combined K + P in APP/PS1 mice at multiple ages. Our findings show that: 1) a single injection of K + P enhances memory retrieval in APP/

(See figure on next page.)

Fig. 5 Chronic, combined (*R,S*)-ketamine + prucalopride administration rescues hippocampal GFAP expression in 6-month-old female APP/PS1 mice. **A-B** Representative image of GFAP immunostaining (green) with Hoechst (blue) in 6-month-old mice. Inset reveals close-up of hippocampal GFAP expression. **C** In male HPC, P increased GFAP in Ctrl mice relative to Sal-administered Ctrl and P-administered APP/PS1 mice. K increased GFAP relative to Sal-administered mice. **D** In female HPC, GFAP was increased in Sal-administered APP/PS1 mice relative to Sal-administered Ctrl mice; this increase in GFAP was rescued by chronic K + P. P-administered APP/PS1 mice exhibited increased GFAP relative to its respective Ctrl group. **E** In male Ctrl mice, P administration increased GFAP expression in the DG-sg. **F** In female mice, there was a significant effect of Drug, but not Genotype or a Genotype x Drug in the DG-sg. **G** In DG-mo of male APP/PS1 mice, K increased GFAP expression relative to Sal-administered APP/PS1 mice and K-administered Ctrl mice. **H** In female DG-mo, APP/PS1 Sal-administered mice exhibited increased GFAP relative to Sal-administered Ctrl mice, which was rescued in K + P-administered APP/PS1 mice. P-administered APP/PS1 mice ad increased GFAP relative to its respective Ctrl mice. **I** In SLM, P-administered Ctrl male mice had increased GFAP expression in comparison to Sal-administered Ctrl mice and P-administered APP/PS1 mice. **J** In female SLM, there was a significant effect of Drug, but not Genotype or Genotype x Drug. **K** In male CA1, P-administered Ctrl male mice had increased GFAP expression in comparison to Sal-administered Ctrl mice and P-administered APP/PS1 mice. In APP/PS1 mice, K increased GFAP relative to Sal. **L** In female CA1, K-administered APP/PS1 mice had increased GFAP relative to K-administered Ctrl mice and Sal-administered APP/PS1 mice. ($n = 3$–6 mice per group). Error bars represent ± SEM. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. HPC, hippocampus; DG-mo, dentate gyrus molecular layer; DG-sg, dentate gyrus granule cell layer; SLM, stratum lacunosum-moleculare; CA1, field CA1 stratum radiatum; μm, microns; GFAP, glial fibrillary acidic protein; Ctrl; control; AD, Alzheimer's disease; Sal, saline; K, (*R,S*)-ketamine; P, prucalopride; K + P, (*R,S*)-ketamine + prucalopride

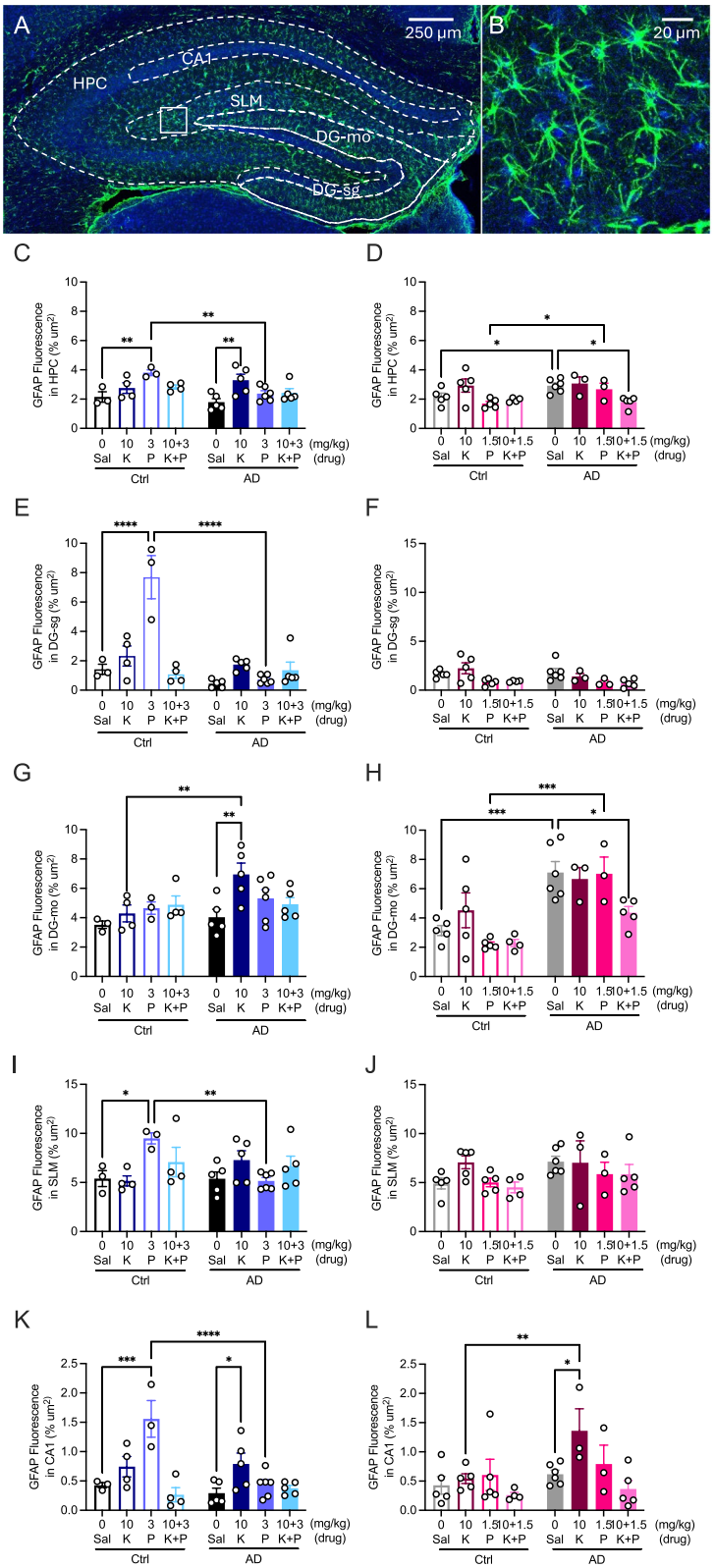

Fig. 5 (See legend on previous page.)

PS1 male mice; 2) when K is combined with P, the dose necessary for efficacy is decreased (10 versus 30 mg/kg), suggesting that P may enhance the pharmacological effects of K; 3) chronic administration of K + P enhances memory retrieval in 2- and 6-month-old APP/PS1 male mice; 4) at the doses tested, single or chronic K + P does not alter memory retrieval in female mice; and 5) chronic K + P treatment rescues hippocampal GFAP expression in APP/PS1 female mice. Overall, our results suggest that adjunctive administration of (*R*,*S*)-ketamine, an NMDAR antagonist, and prucalopride, a 5-HT_4R agonist, enhances memory retrieval in male APP/PS1 mice; therefore, the combination of K + P may provide a potential therapeutic strategy for the treatment of dementia in AD.

In a previous study, APP/PS1 mice exhibited disturbances in sleep measures, such as lower activity, delta power, and deficits in rapid eye movement (REM) sleep [64]. However, in our findings, male and female APP/PS1 mice exhibited comparable sleep patterns to Ctrl. These results may be due to methodological differences in sleep measurements. Notably, our method of Piezo sleep boxes infers sleep patterns using temperature and activity sensors, which may be too general to detect subtle changes in sleep patterns. Using more sensitive methods to record and monitor sleep patterns, such as electroencephalography (EEG) or electromyography (EMG), could be necessary for more discrete measurements of sleep in the brain. Additionally, as our APP/PS1 mice are on a 129S6 background, changes in sleep/wake patterns or activity between mouse strains could change experimental findings [65].

In both sexes, there was no overall effect of Genotype in freezing between Ctrl and APP/PS1 Sal-administered mice following sleep box testing. The lack of difference between Ctrl and APP/PS1 mice was unexpected and possibly attributable to the single housing necessary for Piezo sleep boxes. Acute social isolation, even for short periods of time, has previously been shown to alter activity levels and enhance corticosterone release, particularly in female mice [66, 67]. This mild stressor could affect fear generalization or memory retrieval, leading to comparable freezing levels between Ctrl and APP/PS1 mice. This hypothesis is supported by our findings in our chronic drug administration experiments, as differences in freezing levels between Ctrl and APP/PS1 groups were observed when sleep testing was not administered. In future studies, behavioral assays assessing spatial, social, or object memory may be useful in testing memory encoding and retrieval without confounding effects of single housing stressors.

Despite these findings, acute and chronic administration of K + P was effective in increasing memory retrieval in male APP/PS1 mice, suggesting that the drug combination enhances memory retrieval. While K is a commonly studied drug in the field of major depressive disorder (MDD), to our knowledge it has not previously been investigated as a potential therapeutic for AD [15, 68]. Conversely, 5-HT_4R agonists, including P, have previously been studied for enhancing cognitive performance in a variety of psychiatric and neurological disorders [19, 20, 31]; P has also been shown to be effective when combined with donepezil, an acetylcholinesterase inhibitor, although it has not previously been studied in combination with K for the treatment of AD [40]. Indeed, combined K + P could work via overlapping mechanisms to provide protection against aging and AD. Both K and P are known to affect various types of memory, likely due to neurobiological alterations in plasticity and neurotrophic factors, such as activation of mammalian target of rapamycin (mTOR) and recruitment of brain-derived neurotrophic factor (BDNF) [9, 10, 12, 31, 32, 34, 69]. In particular, K promotes synaptogenesis and dendritic formation [9, 10, 12, 70], while P, by enhancing cholinergic and glutamatergic neurotransmission [31, 41, 71–73], may support long-term potentiation (LTP), neurogenesis, and reduction in inflammatory mediators [74, 75]. Together, these mechanisms could support memory formation, cognitive flexibility, and neuronal survival, all of which are decreased in aging and AD. Nonetheless, the *combination* of K + P seems to deliver the most consistent and impactful results, particularly following chronic administration in APP/PS1 mice.

Although the exact mechanism by which combined K + P enhances memory retrieval is still unknown, our experiments reveal that chronic administration of K + P, but not either drug alone, may rescue hippocampal GFAP expression in APP/PS1 mice. In our experiments, this effect appeared to be mainly driven by the molecular layer of hippocampal DG. In addition to serving as a potential biomarker for neurodegenerative disease and brain injury [55, 76, 77], GFAP is significantly associated with astrocyte reactivity [55, 78–80]. Notably, amyloid deposition is strongly colocalized with astrocyte reactivity, which may help to play a role in plaque phagocytosis [79]. In AD, this process becomes dysfunctional, particularly as the disease progresses, and contributes to cognitive decline and neuronal damage [81]. GFAP-positive reactive astrocytes may also influence tau tangles, although this effect is suggested to be mediated by beta-amyloid processes [82]. While not previously studied in the context of AD, previous reports indicate that K may normalize astrocyte reactivity that is impaired following exposure to stress [83, 84]. Similarly, findings from the field of diabetes indicate that P may help normalize GFAP expression in the gut [75]. Our findings suggest that only combined K + P, but not either drug alone, may restore GFAP expression in DG-mo to normal control levels. Notably, it is still unclear whether normalizing GFAP

levels after brain injury is beneficial. Astrocytes are known to support neuronal metabolic processes [85–87] as well as enhance and facilitate neurotransmitter release [88, 89]; thus, overactive astrocyte and elevated GFAP levels could impair normal synaptic communication, and normalization of GFAP may thus improve and normalize synaptic connectivity[90, 91]. Previously, it has been shown that rescuing astrocyte activity via a calcium sensor can restore LTP in a mouse model of AD [92]. However, further study is necessary to determine how K+P-induced normalization of GFAP expression and in the HPC may contribute to functional recovery of AD pathology and behavior.

Limitations

Although our findings suggest that K+P is beneficial in male mice, our experiments did not indicate an effect of K+P in improving memory retrieval in female APP/PS1 mice. As AD is a disease with higher prevalence in women [5], it will be crucial in future studies to determine why our results did not show a strong benefit in female mice. Notably, although we did not observe an effect during CFC, K+P restored GFAP expression back to Ctrl levels in female APP/PS1 mice. Thus, although K+P did not appear to affect behavior, it may have still exerted a therapeutic neurobiological impact. GFAP was not analyzed in the acute drug treatment groups, as chronic drug injection is likely to have more significant, sustained effects on GFAP expression. Nonetheless, in future experiments, it will be important to assess changes to hippocampal GFAP in response to acute drug treatment. Our sex-specific findings are not surprising considering previous studies from our lab indicating that K and P, alone and in combination, do not alter freezing in female mice [23, 24, 48]. Indeed, as we have previously speculated, male and female mice exhibit distinct behaviors during fear learning and recall [93–96]. Additionally, males and females may process fear-related learning using distinct neural circuits and/or strategies [97–100]. These findings suggest that using different measures of learning and memory, such as spatial, object, or social memory tasks, may be necessary to properly assess memory retrieval in female mice.

It is also possible that sex differences in the pharmacokinetics and/or pharmacodynamics of drug metabolism affected our findings. In female mice, greater levels of K are found in the brain and plasma 5 and 10 min post-injection relative to male mice, regardless of estrous phase [101]. In a pharmacological study examining P administration in humans, men exhibited lower peak plasma concentration of P when compared to women, but these findings were not considered clinically relevant [102]. As we previously determined optimal dose concentrations for K, P, and K+P [23, 24, 48, 103] in both male and female mice, it is unlikely that the different doses used present a confound in our study. Nonetheless, future experiments investigating K+P for the use of AD will necessitate thorough examination of the sex-specific pharmacokinetic and pharmacodynamic profile of K+P.

In our experiments, we used a racemic mixture of K throughout the study. However, emerging evidence suggests that the two enantiomers of K, (*S*)-ketamine and (*R*)-ketamine, exhibit distinct actions in the brain and on behavior [104]. Although (*S*)-ketamine is known to exhibit significantly higher affinity for NMDARs than (*R*)-ketamine, both compounds, and their stereospecific metabolites, have been shown to exhibit beneficial antidepressant-like effects [48, 105–121]. Additionally, both K enantiomers have been separately proposed as potential treatments for AD and other neurogenerative diseases [122–124]. However, it is possible that despite their distinct actions in the brain, both (*S*)- and (*R*)-ketamine may elicit similar or overlapping mechanisms on brain function, and further study is necessary to determine if one enantiomer may have more beneficial impacts on reducing AD pathology.

Finally, we did not observe significant behavioral changes in 12-month-old mice administered K, P, or K+P. In our study, mice were administered drug for 2 weeks prior to and throughout behavioral testing for a total of 24 days. As this period of drug administration is relatively short, it is possible that drug administration would have to continue for significantly longer to achieve observable changes in behavior. Notably, previous findings indicate that the full process of adult hippocampal neurogenesis (AHN), from proliferation to synaptic integration in the hippocampal circuit, takes 6 to 8 weeks [125, 126]. Thus, 24 days of administration would be too short to observe any behavioral or neuronal changes due to effects on hippocampal activity. While we have shown that even a single administration of K, P, or K+P may alter brain-wide changes in correlated activity in 8-week-old adult male and female mice [23, 24], connectivity and neuronal activity in the brain of aged mice may significantly differ and could require longer periods of drug administration in comparison to younger rodents. Ultimately, further experimentation is necessary to determine whether age-related changes in brain function may alter the effects of combined K+P. It is important to note that amyloid pathology was not measured, and this could also impact the function of K+P in aged mice. Nonetheless, as our other experiments showed that combined K+P was effective in enhancing memory in younger (e.g., 2- and 6-month-old mice), our study reinforces the importance of early detection and treatment for those at risk of developing AD.

Conclusions

In summary, we have demonstrated that combined targeting of the NMDAR and 5-HT_4R using K and P is a promising therapeutic strategy for the treatment of dementia in patients with AD. As K + P was effective in enhancing memory retrieval at multiple ages in mice, this suggests that the drug combination may be appropriate for both preventing disease progression and enhancing cognitive function at later disease stages. Furthermore, we have identified GFAP as a potential target by which K + P may suppress AD pathology and restore normal hippocampal brain function. Ultimately, the present study identifies a promising drug combination for therapeutic use in AD and contributes to advancements in targeted therapies for the treatment of dementia in AD.

Abbreviations

5-HT_4R	Serotonin type IV receptor
AD	Alzheimer's disease
AHN	Adult hippocampal neurogenesis
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CA1	Field CA1 stratum radiatum
CFC	Contextual fear conditioning
CIC	Chronic idiopathic constipation
Ctrl	Control
CUIMC	Columbia University Irving Medical Center
DG	Dentate gyrus
DG-mo	Dentate gyrus molecular layer
DG-sg	Dentate gyrus granule cell layer
EEG	Electroencephalography
EMG	Electromyography
FDA	Food and Drug Administration
FST	Forced swim test
g	Gram
GFAP	Glial fibrillary acidic protein
HC	Home cage
HPC	Hippocampus
IACUC	Institutional Animal Care and Use Committee
i.p.	Intraperitoneal
K	(*R,S*)-ketamine
kg	Kilogram
K + P	(*R,S*)-ketamine + prucalopride
LTP	Long-term potentiation
MB	Marble burying
MDD	Major depressive disorder
mg	Milligram
mTOR	Mammalian target of rapamycin
NIA	National Institute on Aging
NMDAR	*N*-Methyl-D-aspartic acid receptor
NPS	Neuropsychiatric symptoms
NSF	Novelty-suppressed feeding
NYSPI	New York State Psychiatric Institute
OF	Open field
P	Prucalopride
PS1	Presenilin 1
RE	Re-exposure
REM	Rapid eye movement
RFMH	Research Foundation for Mental Hygiene, Inc.
Sal	Saline
SEM	Standard error of the mean
SLM	Stratum lacunosum moleculare
ZT	Zeitgeiber

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-025-01804-9.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Authors' contributions

BKC, HCH, and CAD conceived of the study. BKC, HCH, AW, LCM, AY, and MJW collected and analyzed data. SJ provided assistance with experimental design. BKC and HCH wrote the manuscript in consultation with RL, SJ, EW, and CAD. RL, SJ, and EW provided intellectual support. CAD led the study.

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Data availability

Correspondence and requests for materials and data are available upon request and should be addressed to Christine Ann Denny.

Declarations

Ethics approval and consent to participate

All procedures were conducted in accordance with the National Institutes of Health (NIH) regulations and by the Institutional Animal Care and Use Committees (IACUCs) of Columbia University Irving Medical Center (CUIMC) and the Research Foundation for Mental Hygiene, Inc. (RFMH) at the New York State Psychiatric Institute (NYSPI).

Competing interests

BKC, HCH, and CAD are named on provisional patent applications for the prophylactic use of (*R,S*)-ketamine, 5-HT_4R agonists, and other compounds against stress-related psychiatric disorders and Alzheimer's disease. SJ is employed by Silo Pharma, and EW is founder and Chief Executive Officer of Silo Pharma. AW, LCM, AY, MJW, and RL have no conflicts of interest to disclose.

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